09/914006

Atty. Docket #: PT 1.1678
(JÜLICH-13)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

INTERNATIONAL APPL. NO.:	PCT/EP00/01405 :	
INTERNATIONAL FILING DATE:	-02/21/2000-:	
APPLICANT:	LOTHAR EGGELING ET AL :	
SERIAL NO:	(To be assigned) :	**ART UNIT:**
FILED:	-HEREWITH- :	**EXAMINER:**
FOR:	"METHOD FOR MICROBIALLY PRODUCING L-VALINE" :	

Commissioner for Patents
Box PCT
Washington, D.C. 20231

"Express Mail" No.: EK219526023 Date: -AUGUST 21, 2001-

I hereby certify that this paper, along with any other paper or fee referred to in this paper as being transmitted herewith, is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10, postage prepaid, on the date indicated above, addressed to Box PCT, Commissioner for Patents, Washington, D.C. 20231

- Amy L. Hamm -
(Typed or printed name of mailing paper or fee)

Amy L. Hamm
(Signature of person mailing paper)

TRANSMITTAL OF APPLICATION PAPERS
TO U.S. DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. §371
(37 CFR 1.494 OR 1.495)

This Transmittal Letter is based upon PTO Form 1390 (as revised in May, 1993).

The above-identified applicant(s) (jointly with their assignee) have filed an International Application under the P.C.T. and hereby submit(s) to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

International Application No. PCT/EP00/01405

PT 1.1678
(JÜLICH-13)

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JC03 Rec'd PCT/PTO 21 AUG 2001

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.

2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.

3. [X] This is an express request to begin national examination procedures (35 U.S.C. §371[f]) at any time rather than delay.

4. [X] A proper Demand for International Preliminary Examination (IPE) was made to the appropriate Authority (IPEA) within the time period required.

5. [X] A copy of the International Application as filed (35 U.S.C. §371[c][2]) -- In German
   a. [X] is transmitted herewith (required when not transmitted by International Bureau) -with- (3) sheets of Drawings and (11) Sheets of Sequence Listings. See WIPO Publication WO 00/50624.
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).

6. [X] A (verified) translation of the International Application into the English language will follow.

7. [ ] Amendments to the (specification and) claims of the International Application under PCT Article 19 (35 U.S.C. 371[c][3])
   a. [ ] are transmitted herewith (required if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [ ] have not been made and will not be made.
   e. [ ] will be submitted with the appropriate surcharge.

8. [ ] A translation of the amendments to the claims (and/or the specification) under PCT Article 19 (35 U.S.C. §371[c][3]) is enclosed or will be submitted with the appropriate surcharge.

9. [X] An oath or declaration/power of attorney of the inventor(s) (35 U.S.C. §371[c][4]) will follow.
   [ ] and is attached to the translation of (or a copy of) the International Application.
   [ ] and is attached to the substitute specification.

10. [ ] A translation of at least the Annexes to the IPE Report under PCT Article 36 (35 U.S.C. §371[c][5]) is enclosed.

Items 11. to 16. below concern other document(s) or information included:

11. [X] An Information Disclosure Statement under 37 CFR 1.97 and 1.98 is enclosed.

12. [X] An Assignment for recording and a separate cover sheet in compliance with 37 CFR 3.28 and 3.31 will follow.

13. [X] A FIRST preliminary amendment is enclosed.
    A SECOND or SUBSEQUENT preliminary amendment is enclosed.

14. [ ] A substitute specification (including claims, abstract, drawing) is enclosed.

15. [ ] A change of power of attorney and/or address letter is enclosed.

16. [X] Other items of information:

    [X] This application is being filed pursuant to 37 CFR 1.494(c) or 1.495(c), and any missing parts will be filed before expiration of--

        [ ] 22 months from the priority date under 37 CFR 1.494(c), or

        [X] 32 months from the priority date under 37 CFR 1.495(c).

    [X] The undersigned attorney is authorized by the International applicant and by the inventors to enter the National Phase pursuant to 37 CFR 1.494(c) or 1.495(c).

The following additional information relates to the International Application:

- [X] Receiving Office: EPO
- [X] IPEA (if filing under 37 CFR 1.495): EPO
- [X] Priority Claim(s) (35 USC §§ 119, 365):
  German Appln. 199 07 567.0 filed -February 22, 1999-.
- [X] A copy of the International Search Report is
  - [ ] enclosed.
  - [X] attached to the copy of the International Application.
- [X] A copy of the Receiving Office Request Form is enclosed.
- [X] Form PCT/IPEA/409 in German with (4) sheets of Amended Claims.
- [X] Eight (8) sheets of paper Sequence Listing.

The fee calculation is set forth on the next page of this Transmittal Letter.

FEE CALCULATION SHEET

[X] A check in payment of the filing fee, calculated as follows, is attached (37 CFR 1.492).

Basic Fee.....	$ 860.00
Total Number of claims in excess of (20) times $18.....	-0-
Number of independent claims in excess of (3) times $80 ...X (3) =	240.00
Fee for multiple dependent claims $270.....	-0-
TOTAL FILING FEE...	$1,100.00

Kindly send us the official filing receipt.

The Commissioner is hereby authorized to charge any additional fees which may be required or to credit any overpayment to Deposit Account No. 03-2775. This is a "general authorization" under 37 CFR 1.25(b), except that no automatic debit of the issue upon allowance is authorized. An additional copy of this page is attached.

Respectfully submitted,

By Helena C. Rychlicki

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HCR/alh (5899*13)
Enclosures
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PT 1.1678 (JÜLICH-13)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

LOTHAR EGGELING ET AL	:	
SERIAL NO: (To be assigned)	:	**ART UNIT:**
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- Amy L. Hamm -
(Typed or printed name) of person mailing paper or fee)

Amy L. Hamm
(Signature of person mailing paper or fee)

PRELIMINARY AMENDMENT

Sir:

Prior to the determination of the filing fee and any action on the merits of the accompanying new patent application, kindly amend the application as follows:

In the Claims:

Claim 12, line 1, delete "oder 11" ;

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(JÜLICH-13)

Claim 16, line 1, delete "oder 15" ;

Claim 17, line 2, delete "bis 16" .

R E M A R K S

Claims 12, 16 and 17 have been amended to refer to only one preceding claim. Each of the dependent claims, as amended, now depends on only one preceding claim. Therefore no additional fee is required for multiple dependency.

A prompt, favorable action is solicited.

Respectfully submitted,

CONNOLLY BOVE LODGE & HUTZ LLP

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HCR/alh
(5899*13)

A separate clean copy version of amended claims 12, 16 and 17 and a 'marked-up version' will be submitted with English Translation.

Process for the microbial production of L-valine

The present invention relates to a process for the microbial production of L-valine according to Claims 1 through 13 as well as to transformed cells or microorganisms according to Claims 14 through 17 that can be employed in the process.

The amino acid L-valine is a commercially significant product that is used in animal and human nutrition as well as in medicine. Consequently, there is widespread interest in the creation of improved processes for the production of L-valine.

Valine can be manufactured by means of chemical synthesis or else by means of biotechnological methods by fermenting suitable microorganisms in the appropriate nutrient solutions. The advantage of producing microorganisms by means of biotechnology lies in the formation of the correct stereo-isomeric form, namely the L-form of valine that is free of D-valine.

Various types of bacteria such as, for instance, *Escherichia coli*, *Serratia marcescens, Corynebacterium glutamicum, Brevibacterium flavum* or *Brevibacterium lactofermentum* can produce L-valine when in a nutrient solution that contains glucose. U.S. Pat. No. 5,658,766 shows that, in the case of *Escherichia coli*, an increased formation of L-valine can be achieved through mutation in the aminoacyl-tRNA-synthetase. WO 96 06926 also shows that an increase in the formation of L-valine can be achieved with *Escherichia coli* by means of a liponic acid auxotrophy. EP 0,694,614 A1 describes strains of *Escherichia coli* that are resistant to α-ketobutyric acid and that produce L-valine, L-isoleucine or L-leucine when in a nutrient solution that contains glucose.

EP 0,477,000 discloses that the formation of L-valine can be improved through the mutagenesis of *Corynebacterium* or *Brevibacterium* and through selection for valine resistance. The same European patent specification also reveals that improved L-valine formation can be achieved by selecting *Corynebacterium* or *Brevibacterium* for resistance to various pyruvate analogs, such as β-fluoropyruvate, β-chloropyruvate, β-mercaptopyruvate or trimethyl pyruvate. It is known from Nakayama *et al.* (Nakayama *et al.*, 1961, J. Gen. Appl. Microbiol. Jpn.) that

auxotrophies introduced by means of random mutations can lead to an improved L-valine accumulation.

Moreover, EP 0,356,739 A1 shows that the formation of L-valine is improved by means of the plasmid pAJ220V3 with the amplification of the DNA range that codes for the acetohydroxy acid-synthase (ilvBN, also see Figure 1).

The present invention has the objective of providing new principles for the microbial production of L-valine, particularly by means of coryneform bacteria.

This objective is achieved according to the invention in that the dihydroxy acid-dehydratase (ilvD) activity and/or the ilvD gene expression are intensified in a micro-organism. As an alternative or in combination with this, the acetohydroxy acid-synthase (ilvBN) activity and isomeroreductase (ilvC) activity and/or the ilvBNC gene expression are intensified in a microorganism. The process according to the invention can additionally make use of microorganisms in which the activity of at least one enzyme involved in a metabolic pathway that reduces the formation of L-valine is weakened or eliminated. Thus, for instance, the process according to the invention preferably makes use of microorganisms having a defect mutation in the threonine dehydratase (ilvA) gene and/or a defect mutation in one or more genes of the pantothenate synthesis.

As defined in the invention being claimed, the terms "valine" or "L-valine" refer not only to the free acids but also to the salt thereof such as, for example, the calcium, sodium, ammonium or potassium salt.

The term "intensification" describes the increase in the intracellular activity of the cited enzymes ilvD, ilvB, ilvN and ilvC. In order to increase the enzyme activity, especially the endogenous activity in the microorganism is raised. An increase in the enzyme activity can be attained, for example, by changing the catalytic center so as to increase the substrate conversion or by eliminating the effect of enzyme inhibitors. Increased enzyme activity can also be brought about by increasing the enzyme synthesis, for example, by means of gene amplification or by eliminating factors that repress enzyme biosynthesis. According to the invention, the endogenous enzyme activity is preferably increased by mutation of the corresponding endogenous gene. Such mutations can either be generated randomly by means of classical methods such as, for instance, UV radiation or mutation-triggering chemicals, or else in a targeted

manner by means of genetic-engineering methods such as deletion(s), insertion(s) and/or nucleotide exchange(s).

According to the invention, the gene expression is preferably intensified by increasing the number of genocopies. For this purpose, the gene or the genes is/are incorporated into a gene construct or into a vector that preferably contains the regulatory gene sequences associated with the genes, especially those that intensify the gene expression. Subsequently, a microorganism, preferably *Corynebacterium glutamicum*, is transformed with the corresponding gene constructs.

It was ascertained that the intensified expression of the valine biosynthesis gene ilvD from *Corynebacterium glutamicum*, which codes for the enzyme dihydroxy acid-dehydratase, produces L-valine in an improved manner. According to the invention, in addition to the intensified expression of this gene, the intensified expression of the ilvBN genes that code for the enzyme acetohydroxy acid-synthase and of the ilvC gene that codes for the enzyme isomeroreductase also bring about improved formation of L-valine in *Corynebacterium glutamicum*. An additional improvement of the formation of L-valine is achieved through over-expression of all of the cited genes in *Corynebacterium glutamicum*. The genes or gene constructs can be present in the host organism either in plasmids with different numbers of genocopies or else they can be integrated and amplified in the chromosome.

An additional increase in the gene expression can be brought about – either as an alternative to or combined with an increase in the numbers of genocopies – by intensifying regulatory factors that have a positive effect on gene expression. For instance, regulatory elements can be intensified on the transcription level in that especially intensified transcription signals are employed. Moreover, the promoter and regulation region, which is located upstream from the structure gene, can be mutated. Expression cassettes incorporated upstream from the structure gene act in the same manner. By means of inducible promoters, it is additionally possible to increase the expression over the course of the fermentative formation of L-valine. Moreover, the translation can also be intensified, for example, by improving the stability of the m-RNA. Additionally, genes can be used that code for the corresponding enzyme having a high activity. Alternatively, an over-expression of the genes in question can be achieved by changing the medium composition and culture management. The person

skilled in the art can find instructions on this, among others, in Martin *et al.* (Bio/Technology 5, 137-146 (1987)), in Guerrero *et al.* (Gene 138, 35-41 (1994)), in Tsuchiya and Morinaga (Bio/Technology 5, 428-430 (1988)), in Eikmanns *et al.* (Gene 102, 93-98 (1991)), in European patent specification EP 0,472,869, in U.S. Pat. No. 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid *et al.* (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre *et al.* (Journal of Bacteriology 175, 1001-1007 (1993)) and in the patent application WO 96/15246.

By the same token, all conceivable combinations of the above-mentioned measures can be used to intensify the gene expression.

Microorganisms that can be used in the process according to the invention can produce L-valine from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerin and ethanol. These can be gram-positive bacteria, for instance, of the *Bacillus* genus or coryneform bacteria of the above-mentioned *Corynebacterium* genus or else Arthrobacter. As far as the *Corynebacterium* genus is concerned, special mention was already made of the *Corynebacterium glutamicum* species, which is known in specialized circles for its capability to form amino acids. This species includes wild-type strains such as, for example, *B. Corynebacterium glutamicum* ATCC13032, *Brevibacterium flavum* ATCC14067, *Brevibacterium lactofermentum* ATCC13869, *Brevibacterium thiogenitalis* ATCC19240, *Corynebacterium melassecola* ATCC17965 and others.

In order to isolate the gene ilvD from *Corynebacterium glutamicum* or other genes, first a gene bank is set up. How to set up gene banks is laid down in generally known text books and manuals. Examples of these are the text book by Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie [Genes and clones: an introduction to genetic engineering] (published by Verlag Chemie, Weinheim, Germany, 1990) or the manual by Sambrook *et al.*: Molecular Cloning, A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1989). A known gene bank is the *E. coli* K-12 strain W3110, which was set up by Kohara *et al.* (Cell 50, 495-508 (1987)) in λ-vectors. Bathe *et al.* (Molecular and General Genetics, 252: 255-265, 1996) describe a gene bank of *Corynebacterium glutamicum* ATCC13032, which was set up using the cosmid vector SuperCos I (Wahl *et al.*, 1987, Proceedings of the National

Academy of Sciences USA, 84: 2160-2164) in the *E. coli* K-12 NM554 (Raleigh *et al.*, 1988, Nucleic Acids Research 16: 1563-1575). Plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC19 (Norrander *et al.*, 1983, Gene, 26: 101-106) can also be used to set up a gene bank of *Corynebacterium glutamicum* in *Escherichia coli*. Plasmids such as pJC1 (Cremer *et al.*, Mol. Gen. Genet. (1990) 220: 3221-3229) or pECM2 (Jäger *et al.*, J. Bacteriol. (1992) 174: 5462-54465) can be used to produce a gene bank of *Corynebacterium glutamicum* in *Corynebacterium glutamicum*. Particularly suitable hosts are bacteria strains that are restriction-defective or recombination-defective. An example of this is the strain *Escherichia coli* DH5*amcr*, which was described by Grant *et al.* (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649) or the strain *Corynebacterium glutamicum* R127, which was isolated by Liebl *et al.* (FEMS Lett. (1989) 65: 299-304).

The gene bank is subsequently incorporated into an indicator strain by means of transformation (Hanahan, Journal of Molecular Biology 166, 557-580, 1983) or by means of electroporation (Tauch *et al.*, 1994, FEMS Microbiological Letters, 123: 343-347). The indicator strain is characterized by the fact that it has a mutation in the gene of interest that brings about a detectable phenotype, for example, an auxotrophy. The indicator strains or mutants can be obtained from published sources or strain collections or else, if necessary, can be produced by researchers themselves. Within the scope of the present invention, the *Corynebacterium glutamicum* mutant R127/7 was isolated, which is defective in the ilvD gene that codes for the dihydroxy acid-dehydratase. After transformation of the indicator strain such as, for example, the ilvD mutant R127/7, with a recombinant plasmid that carries the gene of interest such as, for instance, the ilvD gene, and after the expression thereof, the indicator strain becomes prototrophic in terms of the property in question such as, for example, its L-valine-requiring characteristics.

The gene or DNA fragment thus isolated can be characterized by the determination of the sequence such as described, for instance, by Sanger *et al.* (Proceedings of the National Academy of Sciences the United States of America, 74: 5463-5467, 1997). Subsequently, the degree of identity to known genes that are stored in databases such as, for example, the GenBank (Benson *et al.*, 1998, Nucleic Acids

Research, 26: 1-7), can be analyzed by means of published methods (Altschul *et al.*, 1990, Journal of Molecular Biology 215: 403-410).

In this manner, the DNA sequence of *Corynebacterium glutamicum* that codes for the gene ilvD, which is an integral part of the present invention as SEQ ID NO 1. Furthermore, the amino acid sequences of the corresponding enzymes were derived from the present DNA sequence using the methods described above. In SEQ ID NO 2, the resultant amino acid sequence of the ilvD gene product, namely, dihydroxy acid-dehydratase, is prepared.

The thus characterized gene can be subsequently expressed either individually or else in combination with others in a suitable microorganism. A known method to express or over-express genes consists of amplifying them by means of plasmid vectors which can be additionally provided with expression signals. Examples of suitable plasmid vectors are those that can replicate in the corresponding microorganisms. The following vectors are possibilities in the case of *Corynebacterium glutamicum*: pEKEx1 (Eikmanns *et al.*, Gene 102: 93-98 (1991)) or pZ8-1 (European patent specification no. 0,375,889) or pEKEx2 (Eikmanns *et al.*, Microbiology 140: 1817-1828 (1994) or pECM2 (Jäger *et al.*, Journal of Bacteriology 174 (16): 5462-5465 (1992)). Examples of such plasmids are pJC1ilvD, pECM3ilvBNCD and pJC1ilvBNCD. These plasmids are *Escherichia coli / Corynebacterium glutamicum* pendulum vectors that carry the gene ilvD or the gene ilvD together with the genes ilvB, ilvN and ilvC.

The inventors have also found that the intensification of the gene individually or in combination with the genes ilvB, ilvN, ilvC has an advantageous effect in those microorganisms that display a reduced synthesis of the amino acid L-isoleucine. This reduced synthesis can also be achieved through deletion of the ilvA gene that codes for the enzyme threonine dehydratase that is specific for L-isoleucine synthesis.

The deletion can also be carried out through targeted recombinant DNA techniques. By means of these methods, for example, the ilvA gene that codes for threonine dehydratase in the chromosome can be deleted. Suitable methods for this are described by Schäfer *et al.* (Gene (1994) 145: 69-73) or by Link *et al.* (Journal of Bacteriology (1998) 179: 6228-6237). It is also possible to delete just parts of the gene or else the mutated fragments of the threonine dehydratase gene can be

exchanged. Therefore, the deletion brings about a loss of the threonine dehydratase activity. An example of such a mutant is the *Corynebacterium glutamicum* strain ATCC13032ΔilvA, which has a deletion in the ilvA gene.

The inventors have also found that the intensification of the genes ilvD, ilvB, ilvN and ilvC in another combination with the reduced synthesis of D-pantothenate, preferably in combination with further deletion of the ilvA gene, has an advantageous effect on the formation of L-valine in microorganisms, for example, by means of deletions in the panB gene and panC gene. The reduced D-pantothenate synthesis can be achieved by weakening or eliminating the corresponding biosynthesis enzymes or their activities. Examples of these are the enzymes ketopantoate hydroxymethyl transferase (EC 2.1.2.11), ketopantoate reductase, pantothenate ligase (EC 6.3.2.1) and aspartate decarboxylase (EC 4.1.1.11). Mutagenesis processes are a way to eliminate or weaken enzymes and their activities.

These include random processes that make use of chemical reagents such as, for instance, N-methyl-N-nitro-nitrosoguanidine or else UV irradiation to induce mutagenesis with a subsequent search of the desired microorganisms for their D-pantothenate-requiring properties. Processes to trigger mutation and to search for mutants are generally known and can be found, among other sources, in Miller (A Short Course in Bacterial Genetics, A Laboratory Manual and Handbook for *Escherichia coli* and Related Bacteria (Cold Spring Harbor Laboratory Press, 1992)) or in the publication titled "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington, D.C. U.S.A., 1981).

This also includes targeted recombinant DNA techniques. By means of these methods, for example, the genes panB, panC, panE and panD that code for the ketopantoate hydroxymethyl transferase, pantothenate ligase, ketopantoic acid reductase or aspartate decarboxylase can be deleted individually or else together in the chromosome. Suitable methods for this purpose are described by Schäfer *et al.* (Gene (1994) 145: 69-73) and by Link *et al.* (Journal of Bacteriology (1998) 179: 6228-6237). It is also possible to delete only parts of the genes or else to exchange mutated fragments of the ketopantoate hydroxymethyl transferase, pantothenate ligase, ketopantoic acid reductase and aspartate decarboxylase. Thus, deletion or exchange brings about a loss or a reduction of the enzyme activity in question. An example of

such a mutant is the *Corynebacterium glutamicum* strain ATCC13032ΔpanBC, which carries a deletion in the panBC operon.

The microorganisms produced according to the invention can be cultivated either continuously or discontinuously in a batch process or else in a fed batch or repeated fed batch process for purposes of producing the L-valine. A compilation of the known cultivation methods is presented in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology, 1. Introduction to Bioprocess Technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and Peripherals] (published by Vieweg Verlag, Braunschweig/Wiesbaden, Germany, 1994)).

The culture medium to be used has to appropriately meet the demands of the individual microorganisms. Descriptions of culture media for various microorganisms can be found in the publication titled "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., U.S.A., 1981). Sources of carbon can be sugars and carbohydrates such as glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats such as, for instance, soy oil, sunflower oil, peanut oil and coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and linoleic acid, alcohols such as, for example, glycerin and ethanol as well as organic acids such as, for instance, acetic acid. These substances can be employed either individually or in the form of a mixture. Sources of nitrogen can be organic compounds containing nitrogen such as peptones, yeast extract, meat extract, malt extract, corn-steep liquor, soybean meal and urea or else inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used either individually or as in the form of a mixture. Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or else the corresponding salts containing sodium can be used as the phosphorus source. The culture medium also has to contain salts of metals such as, for instance, magnesium sulfate or iron sulfate, which are needed for the growth. Finally, essential growth substances such as amino acids and vitamins can be employed in addition to the above-mentioned substances. The feedstocks cited can be added to the culture in the form of a one-time batch or else fed in a suitable manner during the cultivation.

In order to control the pH of the culture, alkaline compounds such as sodium hydroxide, potassium hydroxide and ammonia or else acidic compounds like phosphoric acid or sulfuric acid are employed in the appropriate manner. Anti-foaming agents such as, for example, fatty acid-polyglycol ester, can be used to control foam formation. In order to maintain the stability of plasmids, suitable, selective-action substances can be added to the medium such as, for instance, antibiotics. To maintain aerobic conditions, oxygen or gas mixtures containing oxygen such as, for example, air, are fed into the culture. The temperature of the culture normally lies between 20°C and 50°C [68°F and 122°F], preferably between 25°C and 45°C [77°F and 113°F]. The culture is continued until a maximum quantity of L-valine has formed. This objective is normally achieved within 10 to 160 hours.

The concentration of L-valine formed can be determined using known methods (Jones and Gilligan (1983) Journal of Chromatography 266: 471-482).

The invention will be elaborated upon below on the basis of the following examples of embodiments.

Example 1: Cloning, sequencing and expressing the ilvD gene from *Corynebacterium glutamicum* that codes for the dihydroxy acid-dehydratase

1. Isolation of an ilvD mutant of *Corynebacterium glutamicum*

The strain *Corynebacterium glutamicum* R127 (Haynes 1989, FEMS Microbiology Letters 61: 329-334) was mutated with N-methyl-N-nitro-N-nitrosoguanidine (Sambrook *et al.*, Molecular Cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press). For this purpose, 5 mL of a *Corynebacterium glutamicum culture* grown overnight was mixed with 250 µL of N-methyl-N-nitro-nitrosoguanidine (5 mg/mL of dimethyl formamide) and incubated for 30 minutes at 30°C [86°F] and at 200 rpm (Adelberg 1958, Journal of Bacteriology 76: 326). The cells were subsequently washed twice with sterile NaCl solution (0.9%). By means of replica plating on CGXII minimal medium plates with 15 g/L of agar (Keilhauer *et al.*, Journal of Bacteriology 175: 5595-5603), mutants were isolated that only grew when L-valine, L-isoleucine and L-leucine were added (0.1 g/L of each).

The enzyme activity of the dihydroxy acid-dehydratase was determined in the raw extract of these mutants. For this purpose, the clones were cultivated in 60 mL of LB medium and centrifuged out in the exponential growth phase. The cell pellet was washed once with 0.05 M potassium phosphate buffer and resuspended in the same buffer. Cell digestion was achieved by means of a 10-minute ultrasound treatment (Branson-Sonifier W-250, manufactured by Branson Sonic Power Co., Danbury, CT, United States). Subsequently, the cell fragments were separated out by a 30-minute centrifugation step at 13,000 rpm and at 4°C [39.2°F], and the supernatant was employed as the raw extract in the enzyme test. The reaction batch of the enzyme test contained 0.2 mL of 0.25 M tris/HCl, pH of 8, 0.05 mL of raw extract and 0.15 mL of 65 mM α,β-dihydroxy-β-methyl valerate. The test batches were incubated at 30°C [86°F]; after 10, 20 and 30 minutes, 200-µL samples were taken each time and their concentration of ketomethyl valerate was determined by means of HPLC analysis (Hara *et al.* 1985, Analytica Chimica Acta 172: 167-173). As can be seen in Table 1, the strain R127/7 does not display any dihydroxy acid-dehydratase activity, in contrast to which the isomeroreductase activity and the acetohydroxy acid-synthase

activity are still present as additional enzymes of the synthesis of the branched-chain amino acids.

Table 1 - Specific activities (µmol/min and mg of protein) of valine biosynthesis enzymes in *Corynebacterium glutamicum* strains.

Strain	Dihydroxy acid-dehydratase	Isomeroreductase	Acetohydroxy acid-synthase
R127	0.003	0.05	0.07
R127/7	0.000	0.06	0.09

2. Cloning of the ilvD gene of *Corynebacterium glutamicum*

Chromosomal DNA from *Corynebacterium glutamicum* R127 was isolated as described by Schwarzer and Pühler (Bio/Technology 9 (1990) 84-87). It was then split with the restriction enzyme Sau3A (manufactured by Boehringer Mannheim, Germany) and separated out by means of sucrose-density-gradient centrifugation (Sambrook *et al.*, Molecular cloning. A laboratory manual (1989) Cold Spring Harbor Laboratory Press). The fraction having the fragment size range from about 6 to 10 kb was employed for ligation with the vector pJC1 (Cremer *et al.*, Molecular and General Genetics 220 (1990) 478-480). For this purpose, the vector pJC1 was linearized and dephosphorylated with BamHI. Five nanograms of this were ligated with 20 nanograms of the cited fraction of the chromosomal DNA, as a result of which the mutant R127/7 was transformed through electroporation (Haynes and Britz, FEMS Microbiology Letters 61 (1989) 329-334). The transformants were tested for their ability to grow on CGXII agar plates without the addition of branched-chain amino acids. Of the more than 5000 transformants tested, 8 clones grew on minimal medium plates following replica plating and two-day incubation at 30°C [86°F]. These clones were employed to make plasmid preparations as described by Schwarzer *et al.* (Bio/Technology (1990) 9: 84-87). Restriction analyses of the plasmid DNA revealed that all 8 clones contained the same plasmid, referred to below as pRV. The plasmid carries an insert of 4.3 kb and was tested by means of retransformation for its capacity to complement the ilvD mutant R127/7. Sub-cloning was utilized to delimit the range

responsible for the complementation of the mutant R127/7 to a 2.9 ScaI/XhoI fragment (Figure 2).

3. Sequencing of the ilvD gene

The nucleic acid sequence of the 2.9 kb ScaI/XhoI fragments was determined by the dideoxy chain-termination method by Sanger *et al.* (Proceedings of the National Academy of Sciences of the United States of America (1977) 74: 5463-5467). An Auto-Read Sequencing Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for this. The gel-electrophoretic analysis was carried out with an automatic laser fluorescence sequencer (ALF) manufactured by Amersham Pharmacia Biotech (Uppsala, Sweden). The nucleotide sequence obtained was analyzed with the HUSAR program package (Release 4.0, EMBL, Cambridge, Great Britain). The nucleotide sequence is designated as ID SEQ NO 1. The analysis revealed an open reading frame of 1836 base pairs that was identified as ilvD gene and that codes for a polypeptide of 612 amino acids, which is designated as SEQ ID NO 2.

4. Expression of the ilvD gene

The plasmid pRV was digested with the restriction enzymes ScaI and XhoI in accordance with the information provided by the manufacturer of the restriction enzymes (Roche, Boehringer Mannheim, Germany). Subsequently, the 2.9 kb ilvD fragment was isolated using ion-exchange columns (Quiagen, Hilden, Germany). The overhanging end of the XhoI section of the isolated fragment was filled up with Klenow polymerase. The vector pJC1 (Cremer *et al.*, Mol. Gen. Genet. (1990) 220: 478-480) was PstI-sectioned, likewise treated with Klenow polymerase and subsequently the fragment and the vector were ligated. The ligation batch was employed to transform (Hanahan, Journal of Molecular Biology 166 (1983) 557-580) the *E. coli* strain DH5αmcr (Grant *et al.*, Proceedings of the National Academy of Sciences of the United States of America, 87 (1990) 4645-4649). Using plasmid preparations of clones (Sambrook *et al.*: Molecular Cloning, A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press), a clone was identified that contained the recombinant plasmid pJC1ilvD. This plasmid was utilized to transform

Corynebacterium glutamicum ATCC13032 by means of electroporation, as described by Haynes *et al.* (1989, FEMS Microbiology Letters 61: 329-334). Subsequently, the dihydroxy acid-dehydratase activity coded by ilvD was determined on the basis of *Corynebacterium glutamicum* ATCC13032 pJC1 and *Corynebacterium glutamicum* ATCC13032pJC1ilvD. For this purpose, the clones were cultivated in 60 mL of LB medium and centrifuged out in the exponential growth phase. The cell pellet was washed once with 0.05 M potassium phosphate buffer and resuspended in the same buffer. Cell digestion was achieved by means of a 10-minute ultrasound treatment (Branson-Sonifier W-250, manufactured by Branson Sonic Power Co., Danbury, CT, United States). Subsequently, the cell fragments were separated out by a 30-minute centrifugation step at 13,000 rpm and at 4°C [39.2°F], and the supernatant was employed as the raw extract in the enzyme test. The reaction batch of the enzyme test contained 0.2 mL of 0.25 M tris/HCl, pH of 8, 0.05 mL of raw extract and 0.15 mL of 65 mM α,β-dihydroxy-β-methyl valerate. The test batches were incubated at 30°C [86°F]; after 10, 20 and 30 minutes, 200-µL samples were taken each time and their concentration of ketomethyl valerate was determined by means of HPLC analysis (Hara *et al.* 1985, Analytica Chimica Acta 172: 167-173). As can be seen in Table 2, the strain *Corynebacterium glutamicum* ATCC13032 pJC1ilvD displays increased dihydroxy acid-dehydratase activity in comparison to the control strain.

Table 2 - Specific activity (µmol/min and mg of protein) of the dihydroxy acid-dehydratase in *Corynebacterium glutamicum* ATCC13032.

Plasmid	Dihydroxy acid-dehydratase
pJC1	0.008
pJC1ilvD	0.050

Example 2: Construction of an ilvA deletion mutant of *Corynebacterium glutamicum*

The internal deletion of the ilvA gene of *Corynebacterium glutamicum* ATCC13032 was carried out by means of the system described by Schäfer *et al.* (Gene 145: 69-73 (1994)) for gene exchange. In order to construct the inactivation vector pK19mobsacBΔilvA, first an internal 241 bp BglII fragment was removed

from the ilvA gene present on an EcoRI fragment in the vector pBM21 (Möckel *et al.* 1994, Molecular Microbiology 13: 833-842). For this purpose, the vector was sectioned with BglII, and religated after separation of the ilvA-internal BglII fragment by means of agarose-gel electrophoresis. Subsequently, the incomplete gene was isolated from the vector as an EcoRI fragment and ligated in the vector pK10mobsacB (Schäfer 1994, Gene 145: 69-73) that had been linearized with EcoRI. The inactivation vector pK19mobsacBΔilvA thus obtained was introduced into the *E. coli* strain S 17-1 by means of transformation (Hanahan 1983, Journal of Molecular Biology 166, 557-580) and transferred to *Corynebacterium glutamicum* ATCC13032 via conjugation (Schäfer *et al.* 1990, Journal of Bacteriology 172: 1663-1666). Kanamycin-resistant clones of *Corynebacterium glutamicum* were obtained in which the inactivation vector was integrated in the genome. In order to select the excision of the vector, kanamycin-resistant clones were plated on LB medium containing sucrose (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press) with 15 g/L of agar, 2% glucose / 10% sucrose and colonies were obtained that had once again lost the vector through a second recombination event (Jäger *et al.* 1992, Journal of Bacteriology 174: 5462-5465). By means of inoculation on minimal medium plates (medium CGXII with 15 g/L of agar (Keilhauer *et al.*, Journal of Bacteriology 175 (1993) 5595-5603)) with and without 2mM L-isoleucine or with and without 50 µg/mL of kanamycin, 36 clones were isolated that were kanamycin-sensitive due to excision of the vector and that were isoleucine-auxotrophic and in which the incomplete ilvA gene (ΔilvA allele) was now present in the genome. A strain was designated as ATCC13032ΔilvA and further employed.

Example 3: Cloning of the genes of the pantothenate biosynthesis panB and panC from *Corynebacterium glutamicum*

Cloning of the operon

Chromosomal DNA from *Corynebacterium glutamicum* ATCC13032 was isolated and sectioned with the restriction endonuclease Sau3A. Following gel-electrophoresis separation, DNA fragments within a size range from 3 to 7 kb or from 9 to 20 kb were extracted and subsequently ligated in the singular BamHI section site of the vector pBR322. Colonies having inserts were isolated on the basis of their sensitivity to tetracycline after inoculation on LB plates with 10 µg/mL of tetracycline. Using plasmid preparations (Sambrook *et al.*: Molecular Cloning, A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press) of pooled clones, 8 plasmid pools were isolated, each of which contained 400 plasmids having an insert size of 9 to 20 kb and 9 plasmid pools, each containing 500 plasmids having an insert size of 3 to 7 kb. The *E. coli* panB mutant SJ2 (Cronan *et al.* 1982, J. Bacteriol. 149: 916-922) was transformed with this gene bank by means of electroporation (Wehrmann *et al.* 1994, Microbiology 140: 3349-3356). The transformation batches were plated directly on CGXII medium (J. Bacteriol. (1993) 175: 5595-5603). Plasmid DNA was isolated (Sambrook *et al.* 1989) from clones that were capable of growing without supplementation with pantothenate and retransformation was employed to obtain 8 clones whose D-pantothenate-requiring properties were confirmed. A restriction mapping was carried out with the 8 clones. One of the vectors examined, referred to below as pUR1, contained an insert of 9.3 kb (Figure 3). The transformation of the *E. coli* panC mutant DV39 (Vallari *et al.* 1985, J. Bacteriol. 164: 136-142) showed that the vector pUR1 was likewise capable of complementing the panC defect of this mutant. A 2.2-kb fragment of the insert of pUR1 was sequenced according to the dideoxy chain-termination method by Sanger *et al.* (Proceedings of the National Academy of Sciences of the United States of America (1977) 74: 5463-5467). The gel-electrophoresis analysis was carried out using an automatic laser fluorescence sequencer (A.L.F.) manufactured by Amersham Pharmacia Biotech (Uppsala, Sweden). The nucleotide sequence obtained was analyzed with the HUSAR program

package (Release 4.0, EMBL, Cambridge, Great Britain). The nucleotide sequence is designated as ID SEQ NO 3. The analysis revealed the identification of two open reading frames. One open reading frame encompasses 813 base pairs and exhibits a high level of homology to the already known panB genes from other organisms. The panB gene from *C. glutamicum* codes for a polypeptide of 271 amino acids (see SEQ ID NO 4). The second open reading frame encompasses 837 base pairs and exhibits a high level of homology to the already known panC genes from other organisms. The panC gene from *C. glutamicum* codes for a polypeptide of 279 amino acids (see SEQ ID NO 5).

Example 4: Construction of a panBC deletion mutant from *Corynebacterium glutamicum*

The system for gene exchange described by Schäfer *et al.* (Gene 145: 69-73 (1994)) was used for the genomic panBC fragment of *Corynebacterium glutamicum* ATCC13032 as well as of *Corynebacterium glutamicum* ATCC1303ΔilvA. In order to construct the deletion vector pK19mobsacBΔpanBC, first the 3.95-kb SspI/SalI fragment with panBC was ligated with pUC18 which had been previously SamI/SalI-sectioned. Subsequently, a 1293-bp EcoRV/NruI fragment was removed from the overlapping area of the panBC gene by means of restriction digestion and religation. In order to allow the subcloning into pK19mobsacB, the deleted panBC area was amplified in pUC18 with the two primers 5'-GAGAACTTAATCGAGCAACACCCCTG, 5'-GCGCCACGCCTAGCCTTGGC-CCTCAA and the polymerase chain reaction (PCR) in order to obtain a 0.5-kb ΔpanBC that carries a SalI or EcoRI section site at the ends. The PCR was carried out according to Sambrook *et al.* (Molecular Cloning, A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press) at an annealing temperature of 55°C [131°F]. The fragment thus obtained was ligated with the vector pK19mobsac that had been previously EcoRI/SalI-sectioned and treated with alkaline phosphatase. The resultant inactivation vector pK19mobsacBΔpanBC was introduced into the *Escherichia coli* strain S 17-1 (Hanahan (1983) J. Mol. Biol. 166: 557-580) by means of transformation and then transferred to *Corynebacterium glutamicum* ATCC13032 (Schäfer *et al.*

(1990) J. Bacteriol. 172: 1663-1666) via conjugation. Kanamycin-resistant clones of *Corynebacterium glutamicum* were obtained in which the inactivation vector was integrated in the genome. In order to select for the excision of the vector, kanamycin-resistant clones were plated on LB medium containing sucrose (Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) Cold Spring Harbor Laboratory Press) with 15 g/L of agar, 2% glucose / 10% sucrose and colonies were obtained that had once again lost the vector through a second recombination event (Jäger *et al.* 1992, Journal of Bacteriology 174: 5462-5465). By means of inoculation on minimal medium plates (medium CGXII with 15 g/L of agar (Keilhauer *et al.*, Journal of Bacteriology 175 (1993) 5595-5603) with and without 2mM L-isoleucine or with and without 50 µg/mL of kanamycin, 36 clones were isolated that were kanamycin-sensitive due to excision of the vector and that were isoleucine-auxotrophic and in which the incomplete ilvA gene (ΔilvA alleles) was now present in the genome. A strain was designated as ATCC13032ΔpanBC. As was described in detail in the example, the panBC deletion was also introduced into ATCC13032ΔilvA so as to yield the strain ATCC13032ΔilvAΔpanBC.

Example 5: Expression of the genes ilvD, ilvBN and ilvC in *Corynebacterium glutamicum*

The genes of acetohydroxy acid-synthase (ilvBN) and of isomeroreductase (ilvC) (Cordes *et al.* 1992, Gene 112: 113-116 and Keilhauer *et al.* 1993, Journal of Bacteriology 175: 5595-5603) and of dihydroxy acid-dehydratase (ilvD) (Example 1) were cloned for expression into the vector pECM3. The vector pECM3 is a derivative of pECM2 (Jäger *et al.* 1992, Journal of Bacteriology 174 (16): 5462-5465), which was created by deletion of the approximately 1 kbp-long BamHI/Bg1II DNA fragment that carries the kanamycin resistance gene.

In the vector pKK5 (Cordes *et al.* 1992, Gene 112: 113-116), the ilvBNC genes were already present in a cloned state in the vector pJC1 (Cremer *et al.* 1990, Molecular and General Genetics 220: 478-480). From this, a 5.7-kb XbaI-ilvBNC fragment was isolated and, together with a 3.1-kb XbaI fragment of the vector pRV, it was introduced into the vector pECM3 that had been linearized with XbaI. In this

process, the ligation batch was transformed into the *E. coli* strain DH5αmcr. The plasmid pECM3ilvBNCD was obtained from one clone.

By means of electroporation (Haynes 1989, FEMS Microbiology Letters 61: 329-334) and selection for chloramphenicol resistance (3 µg/mL), the plasmid pECM3ilvBNCD was introduced into the strain ATCC13032ΔilvA and the strain ATCC13032ΔilvA/pECM3ilvBNCD was obtained.

Example 6: Production of L-valine with various strains of *Corynebacterium glutamicum*

In order to examine their valine formation, the strains listed in Table 4 were pre-cultivated in 60 mL of brain heart infusion medium (Difco Laboratories, Detroit, MI, United States) for 14 hours at 30°C [86°F]. Subsequently, the cells were washed once with 0.9% NaCl solution (w/v) and, with this suspension, 60 mL of CGXII medium were inoculated in such a way that the OD600 amounted to 0.5 The medium was identical to the medium described by Keilhauer *et al.* (Journal of Bacteriology (1993) 175: 5595-5603). For the cultivation of the ΔilvA strains, the medium additionally contained 250 mg/L of L-isoleucine. This is shown in Table 3.

Table 3 – Composition of the medium CGXII.

Component	Concentration
$(NH_4)_2SO_4$	20 g/L
urea	5 g/L
KH_2PO_4	1 g/L
K_2HPO_4	1 g/L
$Mg_2O_4 \cdot 7H_2O$	0.25 g/L
3-morpholinopropane sulfonic acid	42 g/L
$CaCl_2$	10 mg/L
$FeSO_4 \cdot 7H_2O$	10 mg/L
$MnSO_4 \cdot H_2O$	10 mg/L
$ZnSO_4 \cdot 7H_2O$	1 mg/L
$CuSO_4$	0.2 mg/L
$NiCl_2 \cdot 6H_2O$	0.02 mg/L

biotin (pH 7)	0.2 mg/L
glucose	40 g/L
protocatechuic acid	0.03 mg/L

After 48 hours of cultivation, samples were taken, the cells were centrifuged out and the supernatant underwent sterile filtration. The L-valine concentration of the supernatant was determined by means of high-pressure liquid chromatography with integrated pre-column derivatization of the amino acid with o-phthdialdehyde as described by Jones and Gilligan (J. Chromatogr. 266 (1983) 471-482). The results are presented in Table 4.

Table 4 – L-valine production with various *Corynebacterium glutamicum* strains.

C. glutamicum	L-valine (mM)
ATCC 13032	0.5
ATCC 13032 pJC1ilvD	2.2
ATCC 13032 pJC1ilvBNC	20.0
ATCC 13032 pJC1ilvBNCD	26.2
ATCC 13032 ΔilvA	2.7
ATCC 13032 ΔilvA pJC1ilvD	7.0
ATCC 13032 ΔilvA pJC1ilvBNCD	28.5
ATCC 13032 ΔpanBC	8.2
ATCC 13032 ΔilvAΔpanBC	31.1
ATCC 13032 ΔilvAΔpanBC pJC1ilvBNCD	72.7

Patent Claims

1. A process for the microbial production of L-valine in which the dihydroxy acid-dehydratase (ilvD) activity and/or the ilvD gene expression are reinforced in a microorganism.

2. A process for the microbial production of L-valine in which the acetohydroxy acid-synthase (ilvBN) activity and the isomeroreductase (ilvC) activity and/or the ilvBNC gene expression are reinforced in a microorganism.

3. The process for the microbial production of L-valine according to Claims 1 and 2.

4. The process according to one or more of the preceding claims,
characterized in that
the endogenous ilvD activity and/or ilvBNC activity in the microorganism is increased.

5. The process according to Claim 4,
characterized in that
the mutation of the endogenous ilvD gene and/or of the ilvBNC genes serves to generate corresponding enzymes having increased activity.

6. The process according to one or more of the preceding claims, characterized in that the ilvD gene expression and/or the ilvBNC gene expression are intensified by increasing the number of genocopies.

7. The process according to Claim 6, characterized in that in order to increase the number of genocopies, the ilvD gene and/or the ilvBNC genes are incorporated into a gene construct.

8. The process according to Claim 7, characterized in that a microorganism is transformed with the gene construct that contains the ilvD gene and/or the ilvBNC genes.

9. The process according to Claim 8, characterized in that *Corynebacterium glutamicum* is employed as the microorganism.

10. The process according to one or more of the preceding claims, characterized in that a microorganism is employed in which the activity of at least one enzyme that is involved in a metabolic pathway that reduces the formation of L-valine is weakened or eliminated.

11. The process according to Claim 10,
characterized in that
the activity of the enzyme threonine dehydratase (ilvA) that is involved in the synthesis of L-valine is weakened or eliminated.

12. The process according to Claim 10 or 11,
characterized in that
the activity of one or more enzymes that are specifically involved in the synthesis of D-pantothenate is weakened or eliminated.

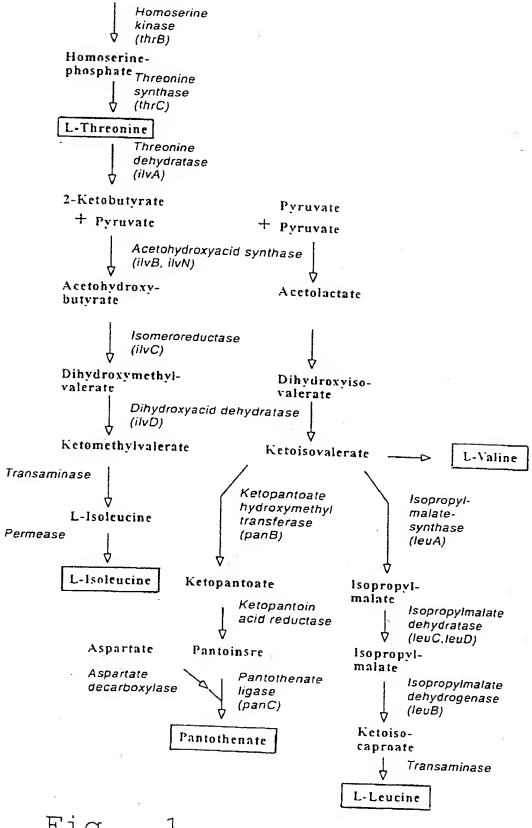
13. The process according to Claim 12,
characterized in that
the activity of the enzyme ketopantoate hydroxymethyl transferase (panB) and/or of the enzyme pantothenate ligase (panC) is weakened or eliminated.

14. A microorganism transformed with a gene construct containing the ilvD gene and/or the ilvBNC genes, in which microorganism the activity of one or more enzymes that are specifically involved in the synthesis of D-pantothenate is weakened or eliminated.

15. The transformed microorganism according to Claim 14 in which the activity of the enzyme ketopantoate hydroxymethyl transferase (panB) and/or of the enzyme pantothenate ligase (panC) is weakened or eliminated.

16. The transformed microorganism according to Claim 14 or 15 in which the activity of the enzyme threonine dehydratase (ilvA) that is involved in the synthesis of L-isoleucine is weakened or eliminated.

17. The transformed microorganism according to one or more of Claims 14 through 16,
characterized by
Corynebacterium glutamicum.


Homoserine kinase (thrB)
Homoserine-phosphate
Threonine synthase (thrC)
L-Threonine
Threonine dehydratase (ilvA)
2-Ketobutyrate
+ Pyruvate
Pyruvate
+ Pyruvate
Acetohydroxyacid synthase (ilvB, ilvN)
Acetohydroxy-butyrate
Acetolactate
Isomeroreductase (ilvC)
Dihydroxymethyl-valerate
Dihydroxyiso-valerate
Dihydroxyacid dehydratase (ilvD)
Ketomethylvalerate
Ketoisovalerate
L-Valine
Transaminase
L-Isoleucine
Permease
L-Isoleucine
Ketopantoate hydroxymethyl transferase (panB)
Isopropyl-malate-synthase (leuA)
Ketopantoate
Isopropyl-malate
Ketopantoin acid reductase
Isopropylmalate dehydratase (leuC,leuD)
Aspartate
Pantoinsre
Isopropyl-malate
Aspartate decarboxylase
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Pantothenate
Ketoiso-caproate
Transaminase
L-Leucine

Fig. 1

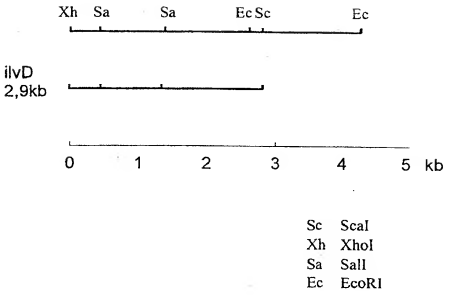
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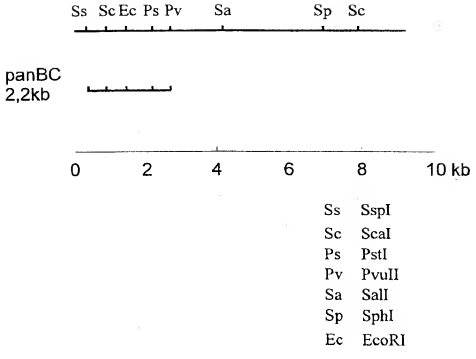
Fig. 2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panBC
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Ss SspI
Sc ScaI
Ps PstI
Pv PvuII
Sa SalI
Sp SphI
Ec EcoRI

Fig. 3

COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney Docket No. 5899*13

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below next to my name,
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **METHOD FOR MICROBIALLY PRODUCING L-VALINE** the specification of which:

(check one) ☐ is attached hereto.

☒ was filed on August 21, 2001 as

Application Serial No. _____ and

was amended on _____
(if applicable)

was amended through _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed
199 07 567.0 (Number)	Germany (Country)	22/2/1999 (Day/Month/Year Filed)	☒ Yes ☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No
(Number)	(Country)	(Day/Month/Year Filed)	☐ Yes ☐ No

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
PCT/EP00/01405	2/21/2000	
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

(Application No.) (filing date)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) associated with the Customer Number provided below to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

In the matter of the above-identified application, please recognize the attorneys associated with **CUSTOMER NUMBER 23416** all of CONNOLLY BOVE LODGE & HUTZ LLP, as attorneys with full power of substitution to prosecute this application and conduct all business in the Patent and Trademark Office connected therewith.

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RESIDENCE		**CITIZENSHIP**
POST OFFICE ADDRESS		
FULL NAME OF FIFTH JOINT INVENTOR IF ANY	**INVENTOR'S SIGNATURE**	**DATE**
RESIDENCE		**CITIZENSHIP**
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